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Short communication

Detection of water-borne *E. coli* O157 using the integrating waveguide biosensor☆

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Abstract

Escherichia coli O157:H7, the most common serotype of enterohemorrhagic *E. coli* (EHEC), is responsible for numerous food-borne and water-borne infections worldwide. An integrating waveguide biosensor is described for the detection of water-borne *E. coli* O157, based on a fluorescent sandwich immunoassay performed inside a glass capillary waveguide. The genomic DNA of captured *E. coli* O157 cells was extracted and quantitative real-time PCR subsequently performed to assess biosensor-capture efficiency. In vitro microbial growth in capillary waveguide is also documented. The biosensor allows for quantitative detection of as few as 10 cells per capillary (0.075 ml volume) and can be used in conjunction with cell amplification, PCR and microarray technologies to positively identify a pathogen.

Keywords: Integrating waveguide biosensor; *E. coli* O157:H7; Sandwich immunoassay; Real-time PCR

1. Introduction

Enterohemorrhagic *Escherichia coli* (e.g., *E. coli* O157:H7) is a major food-borne and water-borne pathogen that causes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Su and Brandt, 1995). Outbreaks have occurred in many developed countries, including Canada, Europe, Australia, and Japan. The Center for Disease Control and Prevention (CDC) estimates that *E. coli* O157:H7 causes nearly 75,000 human infections in the U.S. each year (Mead et al., 1999).

Many methods have been developed to detect *E. coli* O157:H7 in food and water matrices, including traditional culturing with selective media (Hammack et al., 1997; March and Ratnam, 1986), serotyping with specific antibodies to O157 and H7 antigens (Chapman et al., 1997; Czajka and Batt, 1996; Shelton and Karns, 2001; Tomoyasu, 1998), amplification of specific genes by PCR (Higgins et al., 2003; Johnson and Stell, 2001; Maurer et al., 1999; Wang et al., 2002) or hybridization of virulence genes by DNA microarrays (Bekal et al., 2003). Each method has limitations with respect to sensitivity, specificity, and quantitation. Consequently, multiple assays are required to detect and quantify small numbers of water-borne *E. coli* O157:H7 and confirm strain identity.

The integrating waveguide biosensor was originally developed by Ligler et al. (2002) at the Naval Research Laboratory, Washington, DC. The biosensor utilizes a sandwich antibody technique for capture and detection, with the capture antibodies attached to the inner surface of a glass capillary tube. Detection and quantitation are achieved by illuminating the capillary tube (i.e., optical waveguide) at a 90° angle relative to the length of the waveguide and subsequent collection

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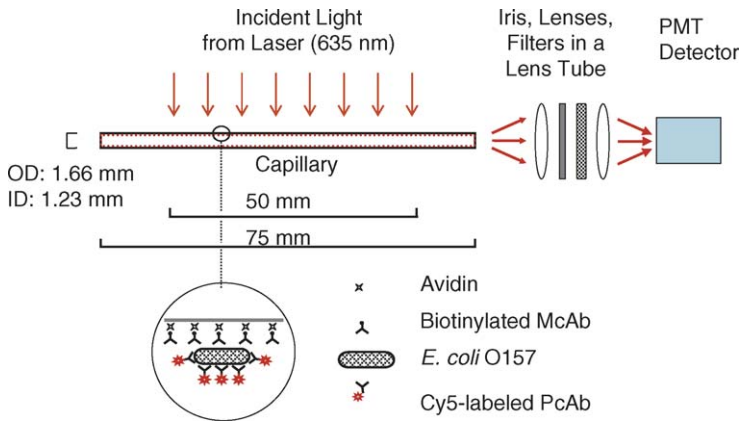

Fig. 1. Side view of waveguide capillary and experimental configuration of the integrating waveguide biosensor. A sandwich immunoassay is performed on the inner surface of capillary using Cy5-labelled anti-*E. coli* O157 antibody for generation of the specific fluorescent signal. Lines with letters 1.66, 75 and 50 mm indicate the diameter, length and laser excitation area of the capillary, respectively.

of the emitted fluorescence from the end of the waveguide (Fig. 1). Initial results gave a detection limit of 40 pg ml^{-1} for mouse IgG and 30 pg ml^{-1} for staphylococcal enterotoxin B (SEB) in the sandwich assays (Ligler et al., 2002), which is more sensitive than other fiber optic and array biosensors (Anderson et al., 1994; Rowe et al., 1999).

Compared to the existing technologies, the integrating waveguide biosensor provides a platform with multiple advantages for the detection of *E. coli* O157:H7 and other analytes in low concentration. Although the capillary tube was originally envisioned only as a waveguide, it is readily available, convenient for laboratory experimentation, and compatible with other sample preparation and detection protocols. The capillary tube can serve as incubation vessel for growth of bacterial pathogens after capture, allowing for confirmation of viability, as well as amplification and retrieval for further characterization. The enclosed structure of capillary tubes is of particular benefit when dealing with pathogenic substances. In addition, clean up of the contaminants by washing, followed with in vitro lysis of pathogens, allows for rapid confirmation of strain identity using PCR or microarray technologies. Since the integrating waveguide biosensor was initially described for the detection of protein targets such as mouse IgG and SEB (Ligler et al., 2002), it remained unclear whether the biosensor could be used for the detection of whole bacterial cells or viral particles. Therefore, we conducted these studies to evaluate the use of the biosensor for the detection of water-borne *E. coli* O157, including assessment of capture efficiency, detection limit, and in vitro growth rates.

2. Materials and methods

2.1. Chemicals and reagents

NeutrAvidin™ was purchased from Pierce Biotechnology (Rockford, IL). One milligram of monoclonal anti-*E. coli* O157 antibody solution (BioDesign, Saco, Maine) was conjugated with Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. One milligram of goat anti-*E. coli* O157:H7 antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) dissolved in 1 ml of phosphate-buffered saline (PBS) was conjugated with Cy5 dye using the FluoroLink-Ab Cy5 labeling kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

2.2. Bacterial strain and growth conditions

E. coli O157 strain 2B, isolated from the Gwynns Falls watershed (Baltimore County, MD), was used for all experiments. This strain has been deposited with the *E. coli* Reference Center, Pennsylvania State University (# 2.4166). The culture was routinely grown at 37 °C in minimal lactose broth medium with yeast extract (MLB-Y) for 16 h (Shelton et al., 2003). At stationary phase the cell concentration was ca. 5×10^8 cells ml^{-1} as determined using a hemocytometer chamber (Hausser Scientific, Gaithersburg, MD). Growth curves in capillary tubes and in 15 × 125 mm test tubes (Kimble/Kontes, Vineland, NJ) containing 10 ml of broth were conducted at 44 °C. Viable cell counts were determined by plating on MacConkey agar using an Autoplate® 4000 (Spiral Biotech, Norwood, MA). After overnight incubation at 37 °C, colonies were counted using a QCount (Spiral Biotech).

2.3. Biosensor sandwich immunoassays

Glass capillary tubes (75 mm long, 1.66 mm O.D., 1.23 mm I.D.) were obtained from Drummond Scientific Company (Broomall, PA). The tubes were prepared according to the procedure described (Ligler et al., 2002). Briefly, capillary tubes were cleaned with methanol/HCl and sulfuric acid, dried with nitrogen, silanized with 3-mercaptopropyl trimethoxysilane in anhydrous toluene under nitrogen, incubated with 4-maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS) (Sigma-Aldrich, St. Louis, MO), and then

treated with NeutrAvidin. The captured antibody was immobilized to the inner surface of the capillary by recirculating biotinylated anti-*E. coli* O157 antibody (10 μg ml^{-1} in PBS containing 0.05% Tween-20; PBST) for 1 h, followed by incubation with 1% BSA to block remaining reactive sites. After rinsing with PBST, capillary tubes were dried by purging with nitrogen gas and stored at 4 °C until use.

2.4. Signal measurement instrument

The optical bench for the capillary immunosensor is basically the same as reported by Ligler et al. (2002) on loan from the Naval Research Laboratory to Creatv MicroTech (Fig. 1). The lenses, optical filters, and the capillary holder are the only components modified. The laser excitation source employed here was a 635 nm, 12 mW diode laser (LaserMax, Bridgewater, NJ). An optical chopper (315 Hz, Stanford Research Systems) was placed in front of the laser. A line generator was used to spread the laser light, thereby providing a beam parallel to the capillary tube. Masking was used to match the laser with 50 mm length of the capillary tube in order to reduce stray light. The laser beam was focused on the capillary by a biconvex lens (20 cm focal length, Newport Corp., Irvine, CA). The inside of the capillary tube was emptied at the time of testing. The capillary tube was held at one end by a simple mechanical support and by an iris on the other end. The iris, at the entrance into a 1 in. lens/filter tube, provided automatic alignment of the capillary tube with the lens/filter set. The lens/filter tube contained collimating lenses, a 670-nm band-pass filter, a 665-nm long pass filter, and a focusing lens. The light out of the focusing lens was input into a Hamamatsu HC-120-05 photomultiplier tube (PMT) module (185–900 nm, 20 kHz, 15 V dc). The PMT output was connected to a SR-510 lock-in amplifier (Stanford Research Systems, Sunnyvale, CA). External reference input was provided from the optical chopper trigger output. The signal from the lock-in amplifier was read manually.

2.5. Real-time PCRs

The sequences of primers and probe for real-time PCR amplification of the *lacZ* gene have been previously described (Higgins et al., 2003). The primers and probe were synthesized by Sigma-Genosys (Houston, TX). The reporter dye FAM (6-carboxyfluorescein) was conjugated at the 5′-end of the probe, and quencher dye, Black Hole quencher (BHQ) dye I was conjugated at the 3′-end. PCR mixture was prepared in a 50-μl volume containing 5 μl of genomic DNA template recovered from capillaries or standard DNA, 0.3 μM (each) primer, 0.1 μM probe, 0.1 μM reference dye ROX, and 25 μl of Brilliant® QPCR Master Mix (Stratagene, La Jolla, CA). The reaction mixture was dispensed into thin-wall PCR tubes and covered with optically clear caps (Stratagene). PCR was performed with the Stratagene MX4000 thermal cycler at the following cycle conditions: denaturation and enzyme activation at 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min, followed by a 5-min extension at 72 °C and holding at 4 °C.

3. Results

3.1. Biosensor capture efficiency

Quantitative real-time PCR was used to determine the capture efficiency for O157 cells by the antibody-coated capillaries. Biosensor capillary tubes with immobilized anti-O157 antibody were incubated statically with different *E. coli* O157 concentrations (10^3, 10^4 and 10^5 cells ml^{-1} in PBS, $n = 5$) at ambient temperature for 1 h, then rinsed with PBST. Capture efficiency was estimated using in vitro lysis of captured cells with 1% Triton X-100 in PBS (37 °C for 10 min) followed by quantitative PCR. Five microliters of lysis solution was used for real-time PCR performed with a Stratagene MX4000 (Stratagene, La Jolla, CA). A calibration curve was first constructed using a 10-fold dilution series of the plasmid standard containing the *lacZ* gene (10^0 to 10^6 copy μl^{-1}). There was a strong linear inverse relationship ($R^2 = 0.9997$) between threshold cycle (CT) and the $\log_{10}$ number of *lacZ* copies over 7 orders of magnitude (Fig. 2). The equation describing the relationship is $CT = -3.2426 \times \log_{10}(lacZ) + 36.879$. The capture efficiency as determined by PCR was 23, 26 and 28% (mean = 26%) for input concentrations of 10^3, 10^4 and 10^5 *E. coli* O157 cells ml^{-1}, respectively.

3.2. Biosensor sandwich assay

The capillary tubes were incubated statically with different *E. coli* O157 concentrations (10^3 to 10^6 cells ml^{-1} in PBS) at ambient temperature for 1 h. After rinsing with PBST, tubes were incubated with 10 μg ml^{-1} of Cy5-labeled polyclonal anti-O157 antibody for 1 h. After rinsing with PBST, fluorescence signals were measured using the integrating waveguide biosensor. Based on the negative control (53.7 ± 5.4 μV), the threshold detection value (average + 3S.D.) was set at 69.9 μV. The input concentration of 10^3 ml^{-1} *E. coli* O157 resulted in a significantly higher signal (94.9 ± 15.7 μV) than that of the negative control (Fig. 3a).

Based on the capillary volume and the capture efficiency, the signal per *E. coli* O157 cell was estimated according to the formula: cells ml^{-1} (input concentration) × 26% (capture efficiency) × 0.075 ml (capillary volume) × 2/3 (ratio of laser excitation area to capillary tube length). The fluorescence signals for estimated *E. coli* O157 number are shown in Fig. 3a. As few as 13 *E. coli* O157 cells per capillary were able to generate a sufficient signal for biosensor detection (94.9 μV). Thus, the biosensor detection limit for *E. coli* O157 appears to be ≤10 cells with an assay time of ca. 3 h. There was a linear relationship ($R^2 = 0.987$) between the logs of fluorescence signal and cells captured, allowing for quantitative detection (Fig. 3b).

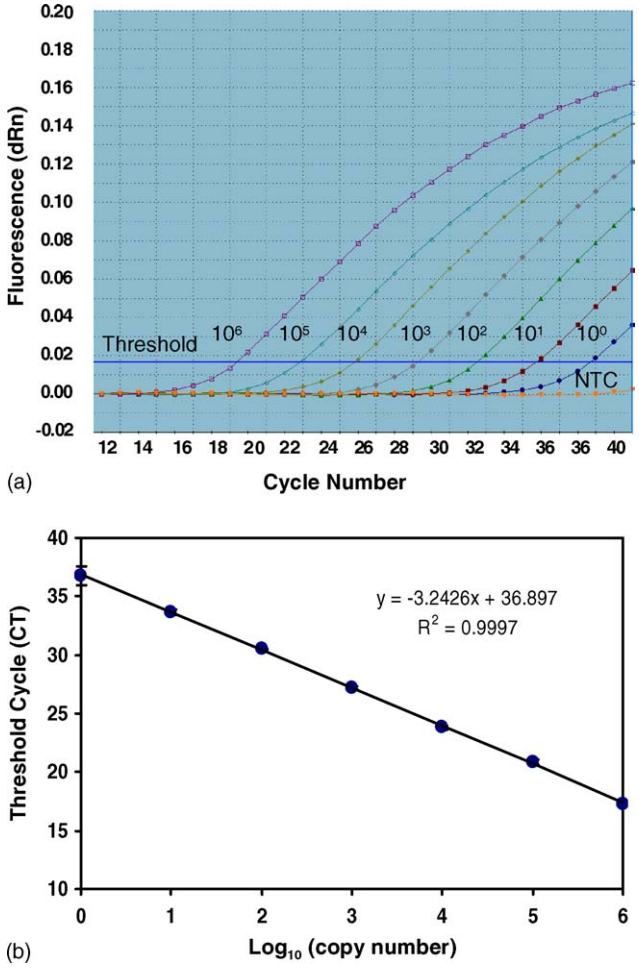

Fig. 2. (a) The plot of real-time PCR amplification of the *lacZ* gene. The *Y*-axis indicates the fluorescence signal while the *X*-axis indicates the amplification cycle. The number shows the copies/μl of the *lacZ* gene in the plasmid standard. Three replicates were performed for each reference DNA sample, but data for only one are shown here. (b) Linear relationship between threshold cycle (CT) and the input copy number ($\log_{10}$) of the *lacZ* gene. Slope, −3.2426; correlation coefficient (R^2), 0.9997. NTC, no-template control; solid line indicates the threshold.

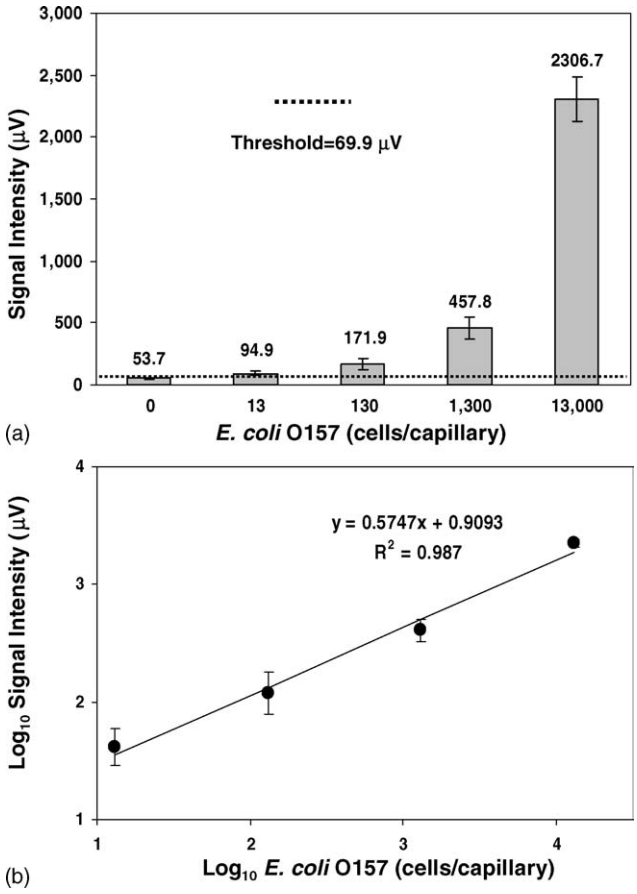

Fig. 3. (a) The relationship between Cy5 fluorescence signal and *E. coli* O157 cell numbers on the capillary. Standard deviations are derived from the mean fluorescence signals of triplicate capillaries. The threshold detection value (mean + 3S.D.) was set at 69.9 μV. (b) Linear relationship between $\log_{10}$ net fluorescence signals and $\log_{10}$ *E. coli* O157 cells per capillary (R^2 = 0.987).

3.3. *E. coli O157 growth curve in biosensor capillary*

Initial growth curves were conducted to assess the effect of volume on growth rate. Capillary (0.075 ml), or test tubes (10 ml) containing ca. 10^4 *E. coli* O157 cells ml^{-1} were filled with MLB-Y medium and test tubes subsampled and capillary tubes sacrificed with time (n = 3). Growth curves in capillary tubes and standard test tubes were essentially identical (Fig. 4a). Therefore, the configuration and volume did not affect cell growth.

To evaluate growth of antibody-immobilized bacteria, capillaries with bound *E. coli* O157 cells (ca. 250 per capillary) were rinsed with PBST and filled with growth medium. At each time point, suspended cells and immobilized cells (cells captured after binary fission) were determined by quantitative real-time PCR. The bacterial growth of suspended cells was also determined by plating and colony counting. Fig. 4b shows growth curves of suspended cells in capillary tubes and antibody-immobilized bacteria bound to the capillary surface. Growth curves for suspended cells using real-time PCR and plating and colony counting were essentially identical (not shown). The immobilized cells on the capillary surface (ca. 14,300) were saturated in 3 h, suggesting that this is the maximum capacity of the capillary. Clearly, the antibody binding of cells to capillary tubes had no adverse impact on subsequent exponential growth. The majority of bacterial cells were in the suspended portion (>96% in 3 h and >99% in 4 h), indicating that the growth medium containing cells from the capillary enrichment procedure was suitable for further genetic or antibiotic assays (Fig. 4b).

4. Discussion

A variety of immunological methods have been described for the capture and detection of *E. coli* O157:H7 using solid supports such as magnetic beads, glass beads, filters, dipsticks, and other materials (Chapman et al., 1997; Czajka

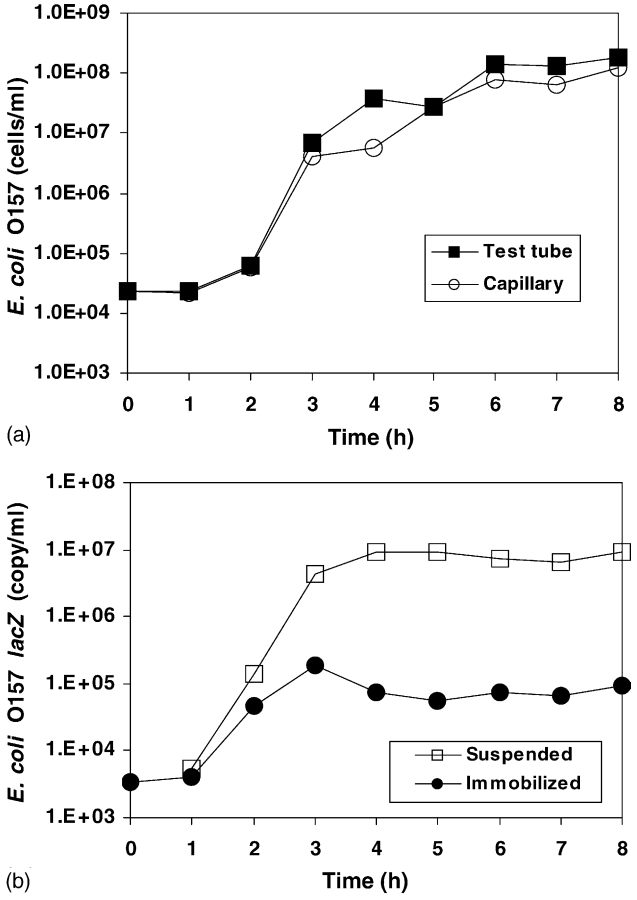

Fig. 4. (a) Growth curve of non-immobilized *E. coli* O157 in the biosensor capillaries (1.66 × 75 mm) and test tubes (15 × 125 mm), three replicates per time point. (b) Growth curve of antibody-immobilized *E. coli* O157 in the biosensor capillary. "Suspended" indicates bacterial cells detected in the medium. "Immobilized" indicates cells captured and re-captured on the capillary surface.

and Batt, 1996; Kim and Doyle, 1992; Pyle et al., 1999; Shelton and Karns, 2001; Tomoyasu, 1998; Weimer et al., 2001). Although conceptually similar, the integrating waveguide biosensor provides comparable or better sensitivity than other methods, with a detection limit of ≤10 *E. coli* O157 cells. In addition, the use of capillary tubes allows for additional assays to be performed after capture and detection. Capillary tubes can serve as culture tubes for growth, allowing for confirmation of viability, as well as amplification and retrieval for further characterization. Cell lysis can also be performed directly in capillary tubes (with or without growth) allowing for strain confirmation using PCR or microarray technologies. A comparable approach was recently described using the Analyte 2000 in conjunction with conventional PCR (Tims and Lim, 2003). However, because of the design of the Analyte 2000, it was necessary to first remove the polystyrene waveguide from the instrument and enrich in a separate culture tube, pellet cells after enrichment, and decant the supernatant to remove inhibitors, and then lyse cells followed by PCR.

Another potential advantage of capillary tubes is the ability to circulate and recirculate relatively large volumes of water, allowing for concentration prior to detection. With other immunological methods, concentration is an independent protocol preceding the immunological assay. Because of the small capillary volume (0.075 ml), the detection limit with static incubation is ca. 10^3 cells ml^{-1}. However, the detection limit can be significantly improved by recirculation and increased capture efficiency (Weimer et al., 2001). Experiments are currently underway to develop a microfluidic chip in flow-though format to enhance sensitivity and reduce analysis time.

5. Conclusion

A novel approach is described for the detection of water-borne pathogens, combining the integrating waveguide biosensor with multiple assays, which allows for determination of bacterial serotype, genotype, and viability. The data presented here demonstrate the concept that the biosensor system is capable of directly capturing *E. coli* O157 from water with subsequent detection in a fluorescence sandwich assay and quantitative real-time PCR. This system can potentially be adapted for the detection of other food- and water-borne pathogens.

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